PCT/GB 2003/005050

RECEIVED
03 FEB 2004
WIPO PCT

INVESTOR IN PEOPLE

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

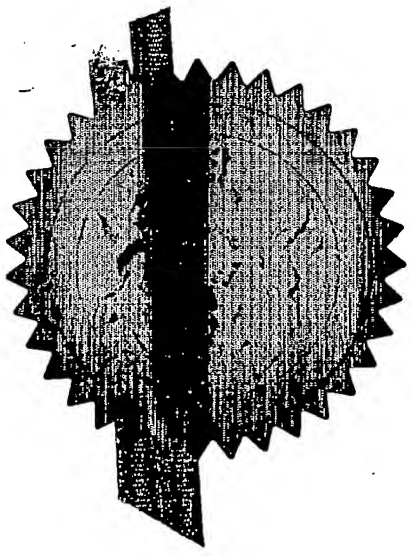
PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.



Signed P. Mahoney

Dated 8 January 2004

21NOV02 E765119-1 D00060
P01/7700 0.00-0227138.5

Patent Form 1/77

Patents Act 1977
(Rule 16)

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

THE PATENT OFFICE
L
20 NOV 2002
LONDON

1/77

The Patent Office

Cardiff Road
Newport
South Wales
NP10 8QQ

1. Your reference	HP/LP6110449.	20 NOV 2002
2. Patent application number *(The Patent Office will fill in this part)*	0227138.5	
3. Full name, address and postcode of the or of each applicant *(underline all surnames)* Patents ADP number *(if you know it)*	NORTHWICK PARK INSTITUTE FOR MEDICAL RESEARCH Harrow Middlesex HA1 3UJ (SEE CONTINUATION SHEET) 8145351001	
If the applicant is a corporate body, give the country/state of its incorporation	GB	
4. Title of the invention	THERAPEUTIC DELIVERY OF CARBON MONOXIDE TO EXTRACORPOREAL AND ISOLATED ORGANS	
5. Name of your agent *(if you have one)*	MEWBURN ELLIS	
"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	YORK HOUSE 23 KINGSWAY LONDON WC2B 6HP	
Patents ADP number *(if you know it)*	109006	

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and (if you know it) the or each application number	Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application	Number of earlier application	Date of filing *(day / month /year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request?
(Answer "Yes" if:
a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))

YES

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	YES
Description	24
Claim(s)	7 DMC
Abstract	1
Drawing(s)	10 + 10

10. If you are also filing any of the following, state how many against each item

Priority documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	1 ✓
Request for preliminary examination and search *(Patents Form 9/77)*	1 ✓
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(Please specify)*	

11. I/We request the grant of a patent on the basis of this application.

Signature	Date
Newburn Ellis	20 November 2002

12. Name and daytime telephone number of person to contact in the United Kingdom — HUGH C E PAGET — 020 7240 4405

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed it it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

a) If you need help to fill in this form or you have any questions, please contact the Patent Office on 08459 500505.

b) Write your answers in capital letters using black ink or you may type them.

c) If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.

d) If you have answered 'Yes' Patents Form 7/77 will need to be filed.

e) Once you have filled in the form you must remember to sign and date it.

f) For details of the fee and ways to pay please contact the Patent Office.

Patents Form 1/77

Patents Act 1977
(Rule 16)

CONTINUATION OF 1/77

The Patent Office

Request for grant of a patent

CONTINUATION SHEET

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

UNIVERSITY OF SHEFFIELD
Firth Court
Western Bank
Sheffield
Yorkshire
S10 2TN

ADP No: 07984004

State of incorporation: GB

Therapeutic Delivery of Carbon Monoxide to extracorporeal and isolated organs

FIELD OF THE INVENTION

The present invention relates to methods of carbon monoxide delivery to isolated organs of humans and other mammals.

BACKGROUND OF THE INVENTION

Transplant surgery is used fairly routinely in cases where patients have body organs that are damaged or malfunctioning. For example heart, lung, liver and kidney transplants are all well known. In transplant surgery, the patient's organ is removed and replaced with an organ donated by a donor. It is often necessary to transport a donated organ from the place of donation to the location of the transplant surgery. This can often involve transport of the donated organ over long distances. A donated organ in transit will be isolated from a blood supply and is therefore subject to ischaemic damage. It is important to limit this ischaemic damage as any damage may affect the functioning of the organ once it has been transplanted.

It is also now common to perform surgery where a body organ, tissue or part is isolated from the patent's blood supply. An example of this is heart valve replacement where the heart is stopped by a cardioplegic solution and the function of the heart is taken over by a mechanical pump system located outside of the body. In this case, the heart is isolated from the patient's blood supply. Again, there is a risk that an organ isolated in such a manner could be affected by ischaemic damage which is undesirable.

It can be seen that a method for limiting ischaemic damage of isolated organs is required.

The beneficial physiological effects of carbon monoxide (CO) have been recognized and reported in a number of publications. A lengthy discussion of the background studies carried out in this area are reported in co-pending application PCT/GB02/02268.

SUMMARY OF THE INVENTION

As exemplified by the experimental data detailed below, the present inventors have found that metal carbonyl compounds can be used to deliver CO to an extracorporeal or isolated organ so as to reduce ischaemic damage of the organ tissue.

Accordingly, in a first aspect, the present invention provides a method of isolated organ treatment comprising contacting the organ with a composition including a metal carbonyl compound or pharmaceutically acceptable salt thereof and at least one pharmaceutically acceptable carrier wherein the metal carbonyl makes available carbon monoxide (CO) to limit post-ischaemic damage. Preferably, the metal carbonyl makes CO available by at least one of the following means:

1) CO derived by dissociation of the metal carbonyl is present in the composition in dissolved form;

2) on contact with a solvent or ligand the metal carbonyl releases CO;

3) on contact with a tissue, organ or cell the metal carbonyl releases CO;

4) on irradiation, the metal carbonyl releases CO.

The term 'isolated organ' is intended to refer to an organ which is isolated from the blood supply. The isolated organ may be extracorporeal e.g. a donated organ outside of the donor's body or it may be intact in a patient's body and isolated from the blood supply for surgical purposes.

The organ may be, for example, a circulatory organ, respiratory organ, urinary organ, digestive organ, reproductive organ, neurological organ, muscle or skin flap or an artificial organ containing viable cells. Most preferably, the organ is a heart, lung, kidney or liver. The "contacting" can be achieved by any method that exposes the organ to the composition e.g. bathing or pumping. Preferably, an isolated organ which is attached to the body i.e. a bypassed organ is perfused with the composition. An isolated organ which is extracorporeal is preferably bathed in the composition.

The term "compound" includes species generated on dissolution.

Certain metal carbonyl compounds are capable of releasing CO on contact with a suitable solvent. The solvent may form a component part of the composition. Thus in this aspect of the invention, the treatment uses CO derived from the metal carbonyl in dissolved form. The conditions under which the carbonyl compound is dissolved in the solvent during preparation of the composition may be controlled such that the CO thus released is retained in solution. This may be facilitated where an equilibrium exists between the dissociated components and the undissociated carbonyl.

The dissociated components of the parent carbonyl may themselves be metal carbonyl complexes capable of releasing further CO. For example, when $[Ru(CO)_3Cl_2]_2$ is

dissolved in DMSO, CO is liberated into solution, and a mixture of tri-carbonyl and di-carbonyl complexes is formed, and these themselves may be capable of releasing further CO.

Release of CO from the complex can be stimulated by reaction with a ligand in solution which for example replaces one of the ligands of the complex leading to loss of CO from the complex. The ligand may be one containing sulphur or nitrogen. Some metal carbonyls may release CO on contact with biological ligands such as glutathione or histidine.

In a further aspect of the invention, the composition may not itself contain dissolved CO, but may be prepared such as to release CO on contact with a suitable solvent or medium. For example, the composition may contain a metal carbonyl compound capable of releasing CO on contact with, for example, water, cardioplegic fluids or perfluorocarbon type blood substitutes.

Alternatively, the composition may be intended to be dissolved in water prior to administration. Such compositions may be prepared in solution or in solid form, such as in tablet form. If they are in solution form, they will typically be prepared in a solvent which does not support dissociation of the metal carbonyl compound, such that release of CO takes place only on contact with the appropriate substance.

In another aspect of the invention the composition may contain a metal carbonyl compound which releases CO on contact with a tissue, organ or cell. It is known that certain metal carbonyl compounds do not release CO to solution but are nevertheless capable of releasing CO to physiological cellular materials or tissues, such as

vascular endothelium. For example, $[Fe(SPh)_2(2,2'$-bipyridine)$(CO)_2]$ is known not to release CO to myoglobin in solution, but is nevertheless capable of promoting dilatation of pre-contracted aortic rings. Without wishing to be limited by any particular theory, it is thought that CO may be released from such compounds as a result of an oxidation-reduction reaction, mediated by cellular components such as cytochromes.

However the invention is not limited to a redox reaction as a mechanism for CO release, since loss of at least a first CO from the complex may occur without redox.

As yet another alternative, the metal carbonyl compound may release CO on irradiation. The compound may be irradiated prior to administration, for example to produce a solution of dissolved CO, or may be irradiated *in situ* after administration. It is contemplated that such compositions may be used to provide controlled, localised release of CO. For example, a pharmaceutical composition of this type may be administered and CO released specifically at a site in need thereof, e.g. to induce vasodilation, by localised irradiation by means of a laser or other radiant energy source, such as UV rays.

Typically the compositions of the present invention release CO such as to make it available to the isolated organ in dissolved form. However, in some circumstances CO may be released from a metal carbonyl directly to a non-solvent acceptor molecule.

It will be apparent that compositions according to the present invention may be capable of delivering CO through one or more of the above described modes of action.

Typically the metal carbonyl compound comprises a complex of a transition metal, preferably a transition metal from groups 6 to 10 (in this specification the groups of the periodic table are numbered according to the IUPAC system from 1 to 18). The number of carbonyl ligands is not limited, provided at least one carbonyl ligand is present. The preferred metals are transition metals of lower molecular weight, in particular Fe, Ru, Mn, Co, Ni, Mo and Rh. Two other metals which may be used are Pd and Pt. In the metal carbonyl complexes used in the invention, the metal is typically in a low oxidation state, i.e. O, I or II. For the metals preferred, the oxidation states are typically not higher than Fe^{II}, Ru^{II}, Mn^{I}, Co^{II} or Co^{III} preferably Co^{I}, Rh^{III} preferably Rh^{I}, Ni^{II}, Mo^{II}. The metal is preferably not a radionuclide. Fe is one particularly suitable metal, since Fe is present in quantity in mammals.

The metal carbonyl compounds may be regarded as complexes, because they comprise CO groups coordinated to a metal centre. However the metal may be bonded to other groups by other than coordination bonds, e.g. by ionic or covalent bonds. Thus groups other than CO which form part of the metal carbonyl compound need not strictly be "ligands" in the sense of being coordinated to a metal centre via a lone electron pair, but will be referred to herein as "ligands" for ease of reference.

The carbonyl compound preferably comprises at least one modulatory ligand. By this is meant a ligand which is not CO, but which modulates a particular property of the complex, such as the tendency to release CO, solubility, hydrophobicity, stability, electrochemical potential, etc. Thus suitable choices of ligand may be made in order to modulate the behaviour of the compound.

For example it may be desirable to modulate the solubility of the compound in organic and/or aqueous solvents, its ability to cross cell membranes, its rate of release of CO on contact with a particular solvent or cell type, etc.

Such ligands are typically neutral or anionic ligands, such as halide, or derived from Lewis bases and having N, P, O, S or C as the coordinating atom(s). Preferred coordinating atoms are N, O and S. Examples include, but are not limited to, sulfoxides such as dimethylsulfoxide, natural and synthetic amino acids and their salts for example, glycine, cysteine, and proline, amines such as NEt_3 and $H_2NCH_2CH_2NH_2$, aromatic bases and their analogues, for example, bi-2,2'-pyridyl, indole, pyrimidine and cytidine, pyrroles such as biliverdin and bilirubin, drug molecules such as YC-1 (2-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole), thiols and thiolates such as EtSH and PhSH, chloride, bromide and iodide, carboxylates such as formate, acetate, and oxalate, ethers such as Et_2O and tetrahydrofuran, alcohols such as EtOH, and nitriles such as MeCN. Particularly preferred are coordinating ligands, such as amino acids, which render the carbonyl complex stable in aqueous solution. Other possible ligands are conjugated carbon groups, such as dienes. One class of ligands which can provide metal carbonyl compounds of use in this invention is cyclopentadienyl (C_5H_5) and substituted cyclopentadienyl. The substituent group in substituted cyclopentadienyl may be for example an alkanol, an ether or an ester, e.g. $-(CH_2)_nOH$ where n is 1 to 4, particularly $-CH_2OH$, $-(CH_2)_nOR$ where n is 1 to 4 and R is hydrocarbon preferably alkyl of 1 to 4 carbon atoms and $-(CH_2)_nOOCR$ where n is 1 to 4 and R is hydrocarbon

preferably alkyl of 1 to 4 carbon atoms. The preferred metal in such a cyclopentadienyl or substituted cyclopentadienyl carbonyl complex is Fe. Preferably the cyclopentadienyl carbonyl complex is cationic, being associated with an anion such as chloride.

CO is suggested to act at least in part through the stimulation of guanylate cyclase activity. Thus the metal carbonyl compound may desirably comprise ligands which modulate the effect of CO on guanylate cyclase. For example, the drug YC-1 (3-(5'-hydroxymethyl-2'-furyl)-1-benzylindole) is thought to enhance stimulation of guanylate cyclase by CO. Thus incorporation of ligands such as YC-1 or derivatives thereof into the metal carbonyl compounds can alter or enhance the biological effects of the released CO.

The metal carbonyl compound may further comprise a targeting moiety, to facilitate release of CO at an appropriate site. The targeting moiety is typically capable of binding a receptor on a particular target cell surface, in order to promote release of CO at the required site. The targeting moiety may be a part of a modulating ligand capable of binding to a receptor found on the surface of the target cells, or may be derived from another molecule, such as an antibody directed against a particular receptor, joined to the complex by a suitable linker.

In most preferred embodiments, the treatment uses a composition for delivery of CO, comprising as active ingredient a compound of the formula $M(CO)_xA_y$ where x is at least one, y is at least one, M is a metal, A is an atom or group bonded to M by an ionic, covalent or coordination bond but is not CO, and, in the case where y>1, each A may be the same or different, or a

pharmaceutically acceptable salt of such a compound. Typically, M is a transition metal, particularly of groups 6 to 10, and A may be selected from neutral or anionic ligands such as halide or derived from Lewis bases and having N, P, O, S or C as the coordinating atom. Mono-, bi- or poly-dentate ligands may be used. More details of preferred metals and ligands are given above.

The carbonyl complex should be pharmaceutically acceptable, in particular non-toxic or of acceptable toxicity at the dosage levels envisaged.

Most preferably, the treatment uses a metal carbonyl compound of the formula

$M(CO)_x A_y B_z$ where

M is Fe, Co or Ru,

x is at least one,

y is at least one,

z is zero or at least one,

each A is a ligand other than CO and is monodentate or polydentate with respect to M and is selected from the amino acids

alanine
arginine
asparagine
aspartic acid
cysteine
glutamic acid
glutamine
glycine
histidine
isoleucine
leucine
lysine

methionine
phenylalanine
proline
serine
threonine
tryptophan
tyrosine
valine

$[O(CH_2COO)_2]^{2-}$ and

$[NH(CH_2COO)_2]^{2-}$, and

B is optional and is a ligand other than CO

x is preferably 3, y is preferably 1 and z is preferably 1.

The term amino acid here used includes the species obtained by loss of the acidic hydrogen, such as glycinato.

B_z represents one or more optional other ligands. There are no particular limitations on B and ligands such as halides, e.g. chloride, bromide, iodide, and carboxylates, e.g. acetate may be used.

M is selected from Fe, Ru and Co. These metals are preferably in low oxidation states, as described above.

The compositions used the present invention typically comprise a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere unduly with the efficacy of the active ingredient. Examples include St Thomas Hospital solutions, Euro-Collins solutions, University of Wisconsin solutions, Celsior solutions, Ringer Lactate solutions, Bretschneider solutions and perflurorcarbons. More

information can be found in Nydegger *et al*, **Transplant Immunology**, 9 (2002) p 215-225.

The compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included. Pharmaceutically acceptable amounts of other solvents may also be included, in particular where they are required for dissolving the particular metal carbonyl compound contained in the composition. The composition may further comprise pharmaceutically acceptable additives such as suspending agents (e.g. sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g. lecithin or acacia); non-aqueous vehicles (e.g. almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); preservatives (e.g. methyl or propyl-p-hydroxybenzoates or sorbic acid); and energy sources (e.g. carbohydrates such as glucose, fats such as palmitate or amino acid).

The temperature at which the treatment is carried out is preferably between 15 and 37 °C for organs still attached to the body but isolated form the blood supply and between 2 and 10 °C for extracorporeal organs, preferably 4 °C.

The amount of CO delivered in the treatment is preferably a prophylactically effective amount. The actual amount administered, and rate and time-course of administration, will depend on the nature of the organ.

The present invention also provides the use of a metal carbonyl compound as herein described in the manufacture of a medicament for delivering CO to an

isolated organ to reduce ischaemic damage of the organ whilst it is isolated from a blood supply. The organ may be extracorporeal or bypassed.

The present invention also provides a method of calibrating a CO electrode using a buffered solution of a metal carbonyl compound having a predetermined concentration in which the metal carbonyl is dissociated to form carbon monoxide (CO) in dissolved form.

The solution is preferably an aqueous solution.

It is advantageous to measure the concentration of CO present in a solution, for example during perfusion of isolated or bypassed organs. A particularly rapid and convenient way of doing this is by use of a CO-sensing electrode capable of measuring dissolved CO, such as is available from World Precision Instruments Ltd (Stevenage, United Kingdom), catalogue number NS-ISONOP-CO.

At times throughout a perfusion, CO concentration in inlet and outlet fluids is measured, and if necessary, the administered amount of CO-releasing molecule is adjusted.

In order to obtain an accurate indication of CO concentrations, the CO sensing electrode should be calibrated at least before use, and possibly at intervals during the perfusion. As the electrode measures dissolved CO, calibration from CO gas would have to be performed by inaccurate methods such as equilibrating a known volume of water with a known volume of CO gas.

However, a particularly convenient and accurate way of performing calibration is by the use of a soluble CO-releasing molecule, such as tricarbonylchloro(glycinato)ruthenium(II) (CORM-3).

Preferably, a water soluble CO-releasing compound is used.

When added to phosphate buffered saline (PBS), CORM-3 rapidly liberates a reproducible amount of CO. Thus, by dissolving a known molar amount of CORM-3 in a known volume of PBS, a known concentration of CO will be present in solution, and the read-out of the CO sensing electrode can be calibrated accordingly.

An alternative and preferred method is to dissolve a known quantity of a water soluble CO-releasing molecule that is stable in water i.e. does not release CO on dissolution and then, when calibration is required, add a compound (e.g. solvent or ligand) that causes liberation of CO. CORM-3 could be used as it is soluble and stable in water. Pyridine, for example, can then be used to cause liberation of CO when required.

Also included in another aspect of the invention is a receptacle containing a predetermined weight of metal carbonyl for use in the calibration method previously described. Preferably the receptacle is a sachet that is easily tearable by hand to release a predetermined weight of solid metal carbonyl for dissolution. Alternatively, the receptacle may contain a pre-prepared solution of known CO concentration.

Throughout this application, references to medical treatment are intended to include both human and veterinary treatment, and references to pharmaceutical compositions are accordingly intended to encompass compositions for use in human or veterinary treatment.

INTRODUCTION OF THE DRAWINGS

Experimental data illustrating the present invention will now be described by reference to the accompanying figures, in which:

Figure 1A shows the structure of tricarbonylchloro-(glycinato)ruthenium(II) (CORM-3);

Figure 1B shows the deoxy-myoglobin and CO-myoglobin absorption spectra;

Figure 1C shows conversion to MbCO;

Figures 2A, 2B, 2C, 3A, and 3B show the effects of various treatments on isolated, perfused rat hearts;

Figures 4A, B and C show the extent of tissue injury; and

Figures 5A to F show metal carbonyl compounds.

EMBODIMENTS OF THE INVENTION AND EXPERIMENTAL DATA

Reagents and material

Tricarbonyldichloro ruthenium(II) dimer ($[Ru(CO)_3Cl_2]_2$), 5-hydroxynoneate (5-HD), 2,3,5-triphenyltetrazolium chloride (tetrazolium red) and all the other reagents were purchased from Sigma (Poole, Dorset) unless specified otherwise.

Stock solutions of $Ru(CO)_3Cl(NH_2CH_2CO_2)$ (CORM-3) (8 mM) were prepared by solubilizing the compound in distilled water. Decomposed CORM-3 (dCORM-3) was prepared by dissolving CORM-3 in Krebs-Henseleit buffer and allowing the solution to stand overnight (18 h) at room temperature. 2, 3, 5-triphenyl-tetrazolium chloride (tetrazolium red) solution (3% w/v) was prepared freshly in Krebs-Henseleit buffer at the end of each experimental protocol prior to infusion into the isolated heart.

All data are expressed as mean ± s.e.m. Differences between the groups analysed were assessed by the Student's two-tailed *t*-test, and an analysis of variance (ANOVA) was performed where more than two treatments were compared. Results were considered statistically significant at $P<0.05$.

Detection of CO release

The release of CO from CORM-3 or dCOMR-3 was assessed spectrophotometrically by measuring the conversion of deoxymyoglobin (deoxy-Mb) to carbonmonoxy myoglobin (MbCO) as previously described [3]. The amount of MbCO formed was quantified by measuring the absorbance at 540 nm (extinction coefficient = 15.4 M^{-1} cm^{-1}). Myoglobin solutions (66 µmol/L final concentration) were prepared fresh by dissolving the protein in 0.04 M phosphate buffer (pH 6.8). Sodium dithionite (0.1 %) was added to convert myoglobin to deoxy-Mb prior to each reading.

When CORM-3 was prepared in distilled water and then added to the phosphate buffer solution containing Mb, a spectrum characteristic of MbCO was rapidly detected (Figure 1B). The amount of MbCO measured after the reaction revealed that 1 mole of CO was liberated per mole of CORM-3. In fact, as shown in Figure 1C, addition of 40 µM CORM-3 resulted in the formation of 36.4±0.9 µM MbCO. When dissolved in water and left for 24 h at room temperature, CORM-3 retained its full ability to liberate CO as assessed by the conversion of Mb to MbCO (data not shown). In contrast, it was discovered that CORM-3 prepared in Krebs-Henseleit buffer gradually decomposed over time and lost its ability to release CO. As shown in Figure 1B and 1C,

CORM-3 in Krebs-Henseleit buffer left overnight at room temperature (dCORM-3) failed to convert deoxy-Mb to MbCO. These data reveal that CORM-3 prepared in water is relatively stable and that physiological solutions such as Krebs-Henseleit and phosphate buffers favour the release of CO from this metal carbonyl complex.

Isolated Heart preparation

Isolated hearts from male Lewis rats (300-350 g) were perfused according to the Langendorff technique as previously described by our group [4]. Briefly, hearts were rapidly excised and perfused at constant flow (11 ml/min) with Krebs-Henseleit buffer (in mM: 119 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.66 $MgSO_4$, 24.9 $NaHCO_3$, 1.18 KH_2PO_4, 5.55 glucose, 2.00 sodium pyruvate, 0.5 EGTA) bubbled with 95% O_2 and 5% CO_2 at 37°C (pH 7.4). Coronary perfusion pressure (CPP) was continuously measured by a pressure transducer (Grass Instruments, Astromed, RI, USA) connected to the aortic cannula. A latex balloon filled with saline was inserted into the left ventricle through the atrium and connected by a catheter to a second pressure transducer. The balloon was inflated to provide an initial end-diastolic pressure (EDP) of 10 mmHg. Both transducers were connected to a computer and data were acquired with BioPac™ instrumentation and analyzed with the accompanying AcqKnowledge™ software (BIOPAC System Inc.). Left ventricular developed pressure (LVDP), heart rate (HR), maximal contraction (+dP/dt) and relaxation (-dP/dt) rates, CPP and EDP were continuously recorded throughout the period of perfusion.

Ischaemia-reperfusion model

Isolated hearts were allowed to equilibrate at constant flow for 30 min and then made globally ischaemic by interrupting the buffer perfusion. Ischaemic hearts were kept at 37°C in the water-jacketed chamber for 30 min and then reperfused for 60 min. All the hemodynamic parameters were continuously monitored throughout the experimental protocol as reported above. Krebs buffer was collected for 10 min from the pulmonary artery prior to the ischaemic event and in the last 10 min of reperfusion for creatine kinase (CK) analysis. At the end of reperfusion, hearts were stained to assess tissue viability using tetrazolium red. In additional experiments, hearts made ischaemic were infused for the first 10 min of reperfusion with CORM-3 or dCORM-3 (10 μM final concentration) via a syringe pump connected to the side arm of the aortic cannula. To assess a possible role of mitochondrial ATP-dependent potassium channels (K_{ATP}) in cardioprotection mediated by CORM-3, control hearts or hearts receiving CORM-3 were pre-treated for 10 min prior to ischaemia with 5-hydroxydodecanoate (5-HD, 50 μM final concentration), a specific blocker of mitochondrial K_{ATP}.

Determination of Infarct Size and Cardiac Muscle Damage

Hearts from each experimental group (n=5) were stained for tissue viability at the end of the reperfusion period. Hearts were perfused through a side arm of the aortic cannula for 20 min with tetrazolium red (3% w/v) in Krebs Henseleit buffer at 37 °C. The tetrazolium salt stains the viable myocardium brick red, whereas the infarcted tissue remains unstained and appears white. After staining, hearts were removed and

stored in 2% formalin in the dark prior to analysis. Hearts were carefully cut into 2-mm thick sections, scanned into a computer using an AGFA Arcus® II scanner and the total ischaemic size was determined by volumetric analysis software (Scion Image®, Scion Corporation, MA, USA). Cardiac muscle damage was assessed by measuring the release of creatine kinase (CK) into the perfusate using a commercially available spectrophotometric assay kit (DG147-A) from Sigma Diagnostic (Poole, Dorset).

Results

Hemodynamic, biochemical and histological parameters were measured to assess the potential beneficial effects of CORM-3 on the functional recovery of hearts subjected to ischaemia-reperfusion. As shown in Figure 2A, 2B and 2C, the cardiac performance of hearts treated with CORM-3 at reperfusion was significantly higher compared to control hearts (data marked 'CON' in Figures). After 60 min of reperfusion, control hearts displayed a 34% decrease in left ventricular-developed pressure (LVDP) compared to baseline whereas hearts reperfused in the presence of CORM-3 showed a 44% increase in this parameter ($p<0.05$, Figure 2A). This positive inotropic effect mediated by CORM-3 was also evident when analyzing the maximal rate of contraction (+dP/dt) and relaxation (-dP/dt) in post-ischaemic hearts (see Figures 2B and 2C). While no significant changes in +dP/dt and -dP/dt were observed in control hearts after ischaemia-reperfusion, hearts reperfused in the presence of CORM-3 showed a significant increase in both +dP/dt (from 2099±99 to 3726±542 mmHg/s, $p<0.05$) and -dP/dt (from 1432±149 to

2207±258 mmHg/s, $p<0.05$). CORM-3 was also capable of preventing the increases in end diastolic (EDP) and coronary perfusion pressure (CPP) that are typical of post-ischaemic myocardial dysfunction in this model. As shown in Figure 3A and 3B, control hearts showed an increase of 36.9±8.4 mmHg in EDP and 31.6±8.8 mmHg in CPP at the end of reperfusion whereas CORM-3 significantly attenuated these effects (3±1.8 and 13±2.2 mmHg for EDP and CPP, respectively; $p<0.05$). Biochemical and histological analysis confirmed the beneficial effect of CORM-3 in ameliorating the functional recovery of the ischaemic hearts. Creatine kinase (CK) activity, an index of cardiac tissue injury, was elevated in the buffer of reperfused control hearts (from 7.4±3.2 to 60.4±8.0 U/L) but the activity was significantly attenuated in the presence of CORM-3 (from 6.5±2.3 to 19.9±5.3 U/L) ($p<0.05$, see Figure 4A). Similarly, the infarct size measured by staining the myocardial tissue with tetrazolium red at the end of the reperfusion period was significantly ($p<0.05$) reduced in hearts reperfused with CORM-3 (2.3±0.6%) compared to control (9.5±2.1%) (Figure 4B and 4C). It is interesting to note that the cardioprotective action elicited by CORM-3 as observed from all the parameters measured can be attributed to CO being liberated from this metal carbonyl during the reperfusion period. In fact, the negative control dCORM-3, which is incapable of releasing CO (see Figure 1B and 1C), did not promote any protective effect on the hemodynamic, biochemical and histological parameters measured (see Figures 2-4).

Mechanism of cardioprotection by CORM-3: possible involvement of K channels

The potassium ion (K^+) is the major cytoplasmic and mitochondrial cation, and net flux of K^+ across the inner membrane critically regulates mitochondrial activity including regulation of energy production (ATP) and maintenance of calcium homeostasis, which are both essential for cellular survival [1]. The ATP-sensitive K^+ channel (K_{ATP}) has been identified as an important regulator of K^+ flux and the opening of this channel has been implicated in protection of the myocardium against ischaemia-reperfusion [1, 2, 5]. Blockade of K_{ATP} channels with specific inhibitors such as 5-hydroxydodecanoate (5-HD) has been shown to exacerbate myocardial dysfunction and tissue damage during ischaemia reperfusion [5]. CO has also been shown to activate the opening of a different type of K^+ channel that regulate the flux of calcium (K_{Ca}) in smooth muscle cells and mediates vaso-relaxation [6, 7]. Therefore, it was hypothesized that part of the cardioprotective mechanism mediated by CORM-3 could involve the activation of K_{ATP} mitochondrial channels. The data presented in Figure 2, 3 and 4 corroborate this hypothesis. In fact, the protective effects of CORM-3 in preserving myocardial contractility (LVDP, +dP/dt and -dP/dt) and preventing the increases in diastolic and coronary pressures (EDP and CPP) during reperfusion following the ischaemic event are totally abolished by pre-treatment of isolated hearts with 5-HD, an inhibitor of K_{ATP} mitochondrial channel (Figure 2 and 3, respectively). Moreover, the levels of CK in the buffer at the end of reperfusion and the extent of the infarct size in hearts treated with 5-HD and CORM-3 were similar

to control hearts and significantly higher ($p<0.05$) compared to hearts treated with CORM-3 alone (Figure 4). The data indicate that CO released by CORM-3 could facilitate the opening of K_{ATP} channels which are crucial for maintaining cardiac function following ischaemic episodes.

Syntheses

Synthetic methods for obtaining compounds shown in Figures 5a to 5f are disclosed in co-pending application PCT/GB02/02268 the entire contents of which is incorporated herein by reference.

By way of example, the synthesis of $Ru(CO)_3Cl(NH_2CH_2CO_2)$ is set out below. Purity of the product has not been investigated in detail.

Preparation of $Ru(CO)_3Cl(NH_2CH_2CO_2)$ [Mr 294.5]

Glycine complex. Reference number: CO-RM-3

$[Ru(CO)_3Cl_2]_2$ (0.129g, 0.25 mmol) and glycine (0.039g, 0.5 mmol) were placed under nitrogen in a round bottomed flask. Methanol (75 cm^3) and sodium ethoxide (0.034g, 0.50 mmol) were added and the reaction allowed to stir for 18 hours at room temperature. The solvent was then removed under pressure and the yellow residue redissolved in THF, filtered and excess 40-60 light petroleum added. The yellow solution was evaporated down to give a pale yellow solid (0.142g, 96%). The product was stored in closed vials at 4°C.

Alternative, preferred preparation of $Ru(CO)_3CI(NH_2CH_2CO_2)$ [M_R294.6]

Glycine complex. Reference numbers: CORM-3.

$[Ru(CO)_3Cl_2]_2$ (0.129g, 0.25 mmol) and glycine (0.039g, 0.50 mmol) were placed under nitrogen in a round bottomed flask. Methanol (40 cm^3) and sodium methoxide (0.5M solution in MeOH, 1.00 cm^3, 0.50 mmol) were added and the reaction stirred for 18 hours. HCl (2.0 M solution in diethyl ether) was added in small aliquots until the IR band at 1987 cm^{-1} in solution IR spectroscopy could no longer be detected. The solvent was then removed under reduced pressure and the yellow residue redissolved in THF, filtered and an excess of 40-60 light petroleum added. The resulting precipitate was isolated by pipetting off the mother liquor and drying under high vaccum. The same work up was repeated for the mother liquor once concentrated. The colour of the product varied between whit and pale yellow and was produced in an average yield of 0.133 g, (90%).

While the invention has been described in conjunction with the exemplary embodiments described above, many equivalent modifications and variations will be apparent to those skilled in the art when given this disclosure. Accordingly, the exemplary embodiments of the invention set forth above are considered to be illustrative and not limiting. Various changes to the described embodiments may be made without departing from the spirit and scope of the invention.

References

1. Holmuhamedov EL, Jovanovic S, Dzeja PP, Jovanovic A and Terzic A. Mitochondrial ATP-sensitive K+ channels modulate cardiac mitochondrial function. *Am J Physiol* 275: H1567-H1576, 1998.

2. Lawton JS, Hsia PW, McClain LC, Maier GW and Damiano RJ, Jr. Myocardial oxygen consumption in the rabbit heart after ischemia: hyperpolarized arrest with pinacidil versus depolarized hyperkalemic arrest. *Circulation* 96: II-52, 1997.

3. Motterlini R, Clark JE, Foresti R, Sarathchandra P, Mann BE and Green CJ. Carbon monoxide-releasing molecules: characterization of biochemical and vascular activities. *Circ Res* 90: E17-E24, 2002.

4. Motterlini R, Samaja M, Tarantola M, Micheletti R and Bianchi G. Functional and metabolic effects of propionyl-L-carnitine in the isolated perfused hypertrophied rat heart. *Mol Cell Biochem* 116: 139-145, 1992.

5. Wang L, Cherednichenko G, Hernandez L, Halow J, Camacho SA, Figueredo V and Schaefer S. Preconditioning limits mitochondrial Ca^{2+} during ischemia in rat hearts: role of K_{ATP} (channels) . *Am J Physiol Heart Circ Physiol* 280: H2321-H2328, 2001.

6. Wang R and Wu L. The chemical modification of K_{Ca} channels by carbon monoxide in vascular smooth muscle cells. *J Biol Chem* 272: 8222-8226, 1997.

7. Wu L, Cao K, Lu Y and Wang R. Different mechanisms underlying the stimulation of K(Ca) channels by nitric oxide and carbon monoxide. *J Clin Invest* 110: 691-700, 2002.

CLAIMS:

1. A method of isolated organ treatment comprising contacting the organ with a composition including a metal carbonyl compound or pharmaceutically acceptable salt thereof and at least one pharmaceutically acceptable carrier wherein the metal carbonyl makes available carbon monoxide (CO) to limit post-ischaemic damage.

2. A method according to claim 1 wherein said metal carbonyl makes CO available by at least one of the following means:

1) CO derived by dissociation of the metal carbonyl is present in the composition in dissolved form;

2) on contact with a solvent the metal carbonyl releases CO;

3) on contact with a tissue, organ or cell the metal carbonyl releases CO;

4) on irradiation, the metal carbonyl releases CO.

3. A method according to claim 1 or claim 2 wherein said isolated organ is extracorporeal.

4. A method according to claim 1 or claim 2 wherein said isolated organ is inside or attached to the body but isolated from the blood supply.

5. A method according to any one of claims 1 to 4 wherein the contacting step includes perfusing said organ with said composition.

6. A method according to any one of claims 1 to 5 wherein the metal carbonyl is a compound of the formula $M(CO)_xA_y$ where x is at least one, y is at least one, M is a metal, the or each A is an atom or group bonded to M by an ionic, covalent or coordination bond but is not CO, and in the case where y>1 each A may be the same or different, or a pharmaceutically acceptable salt of such a compound.

7. A method according to claim 6 wherein M is a transition metal.

8. A method according to claim 6 or claim 7, wherein A is selected from neutral or anionic ligands such as halide or derived from Lewis bases and having N, P, O, S or C as the coordinating atom.

9. A method according to any one of claims 1 to 5 wherein the metal carbonyl compound has the formula

$M(CO)_x A_yB_z$ where

M is Fe, Co or Ru,

x is at least one,

y is at least one,

z is zero or at least one,

each A is a ligand other than CO and is monodentate or polydentate with respect to M and is selected from the amino acids

alanine

arginine

asparagine

aspartic acid

cysteine

glutamic acid
glutamine
glycine
histidine
isoleucine
leucine
lysine
methionine
phenylalanine
proline
serine
threonine
tryptophan
tyrosine
valine

$[O(CH_2COO)_2]^{2-}$ and

$[NH(CH_2COO)_2]^{2-}$, and

B is optional and is a ligand other than CO.

10. Use of a metal carbonyl compound in the manufacture of a medicament for treatment of an isolated organ to limit post-ischaemic damage in an isolated organ which is inside or attached to the body but isolated from the blood supply.

11. Use according to claim 10 wherein the metal carbonyl is a compound of the formula $M(CO)_xA_y$ where x is at least one, y is at least one, M is a metal, the or each A is an atom or group bonded to M by an ionic, covalent or coordination bond but is not CO, and in the case where y>1 each A may be the same or different, or a pharmaceutically acceptable salt of such a compound.

12. Use according to claim 11 wherein M is a transition metal.

13. Use according to claim 11 or claim 12, wherein A is selected from neutral or anionic ligands such as halide or derived from Lewis bases and having N, P, O, S or C as the coordinating atom.

14. Use according to claim 10 wherein the metal carbonyl compound has the formula

$M(CO)_x A_y B_z$ where

M is Fe, Co or Ru,

x is at least one,

y is at least one,

z is zero or at least one,

each A is a ligand other than CO and is monodentate or polydentate with respect to M and is selected from the amino acids

alanine
arginine
asparagine
aspartic acid
cysteine
glutamic acid
glutamine
glycine
histidine
isoleucine
leucine
lysine
methionine
phenylalanine
proline

serine
threonine
tryptophan
tyrosine
valine
$[O(CH_2COO)_2]^{2-}$ and
$[NH(CH_2COO)_2]^{2-}$, and
B is optional and is a ligand other than CO.

15. A method of calibrating a CO electrode using a buffered solution of a metal carbonyl compound having a predetermined concentration in which the metal carbonyl is dissociated to form carbon monoxide (CO) in dissolved form.

16. A method according to claim 15 wherein the metal carbonyl is a compound of the formula $M(CO)_xA_y$ where x is at least one, y is at least one, M is a metal, the or each A is an atom or group bonded to M by an ionic, covalent or coordination bond but is not CO, and in the case where y>1 each A may be the same or different, or a pharmaceutically acceptable salt of such a compound.

17. A method according to claim 16 wherein M is a transition metal.

18. A method according to claim 16 or claim 17, wherein A is selected from neutral or anionic ligands such as halide or derived from Lewis bases and having N, P, O, S or C as the coordinating atom.

19. A method according to claim 15 wherein the metal carbonyl compound has the formula

$M(CO)_x A_y B_z$ where

M is Fe, Co or Ru,

x is at least one,

y is at least one,

z is zero or at least one,

each A is a ligand other than CO and is monodentate or polydentate with respect to M and is selected from the amino acids

alanine
arginine
asparagine
aspartic acid
cysteine
glutamic acid
glutamine
glycine
histidine
isoleucine
leucine
lysine
methionine
phenylalanine
proline
serine
threonine
tryptophan
tyrosine
valine

$[O(CH_2COO)_2]^{2-}$ and

$[NH(CH_2COO)_2]^{2-}$, and

B is optional and is a ligand other than CO.

20. A method according to any one of claims 15 to 19 wherein said buffered solution is an aqueous solution.

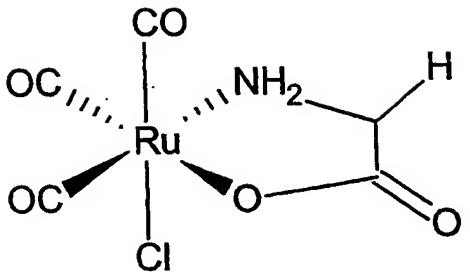
21. A receptacle containing a predetermined weight of a metal carbonyl compound or pharmaceutically acceptable salt for use in the method according to any one of claims 15 to 20.

Therapeutic Delivery of Carbon Monoxide

ABSTRACT

Metal carbonyls are used to deliver CO to isolated organs to limit post-ischaemic damage. The isolated organ may be extracorporeal e.g. for use in a transplant or may be inside or attached to the body but isolated form the blood flow. The carbonyl preferably has one or more other ligands other than CO, such as amino acids, to modulate the CO release property and solubility.

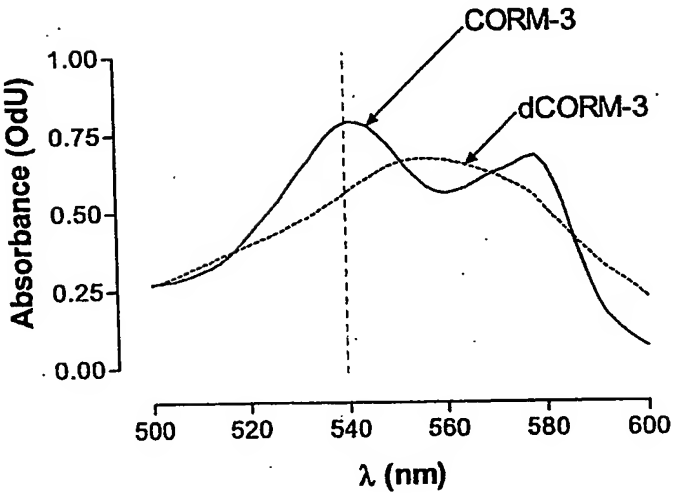
A



Ru(CO)3, Cl– Glycinate

(CORM-3)

B



C

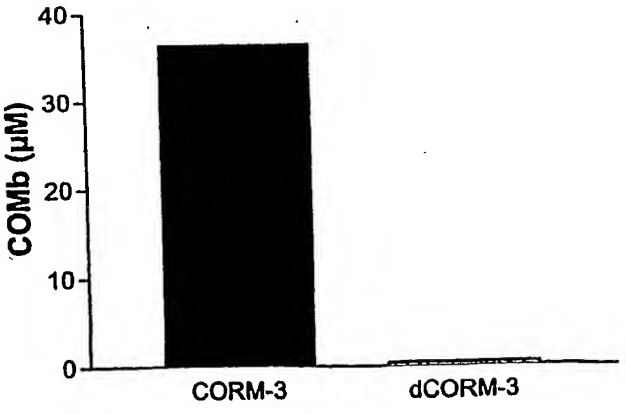
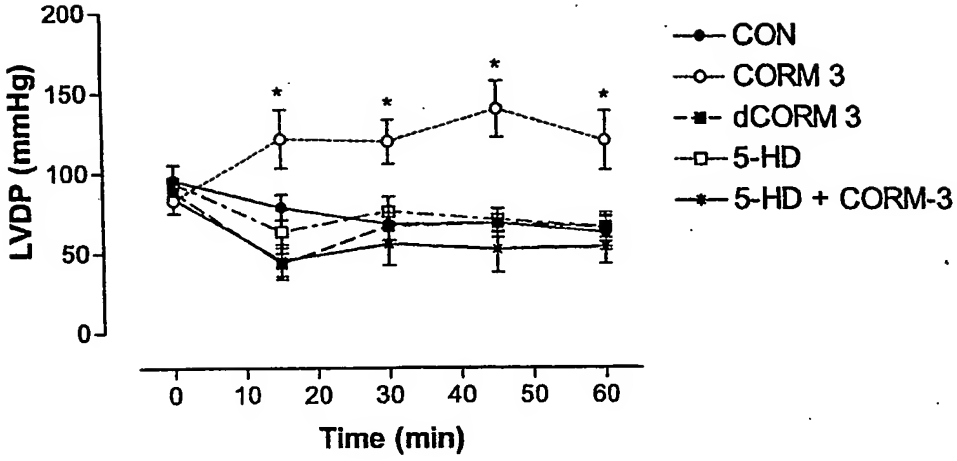
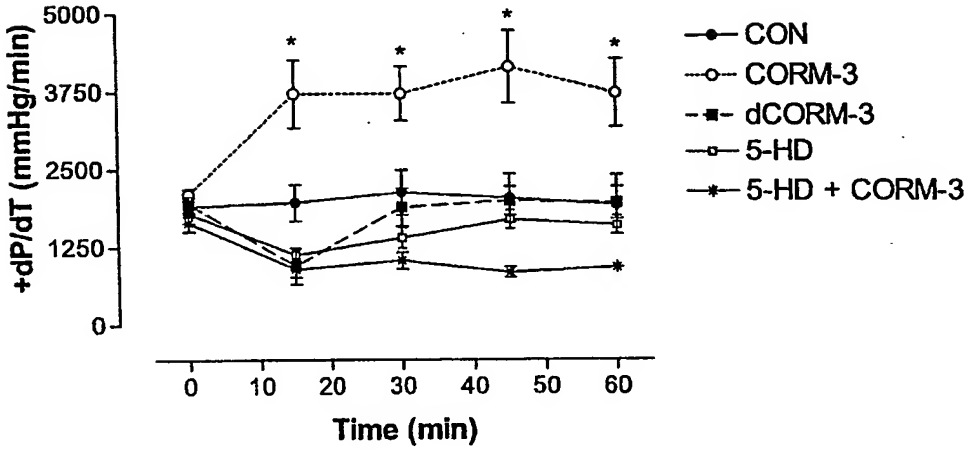


FIGURE 1

A


LVDP (mmHg)
200
150
100
50
0
0 10 20 30 40 50 60
Time (min)
CON
CORM 3
dCORM 3
5-HD
5-HD + CORM-3

B


+dP/dT (mmHg/min)
5000
3750
2500
1250
0
0 10 20 30 40 50 60
Time (min)
CON
CORM-3
dCORM-3
5-HD
5-HD + CORM-3

C

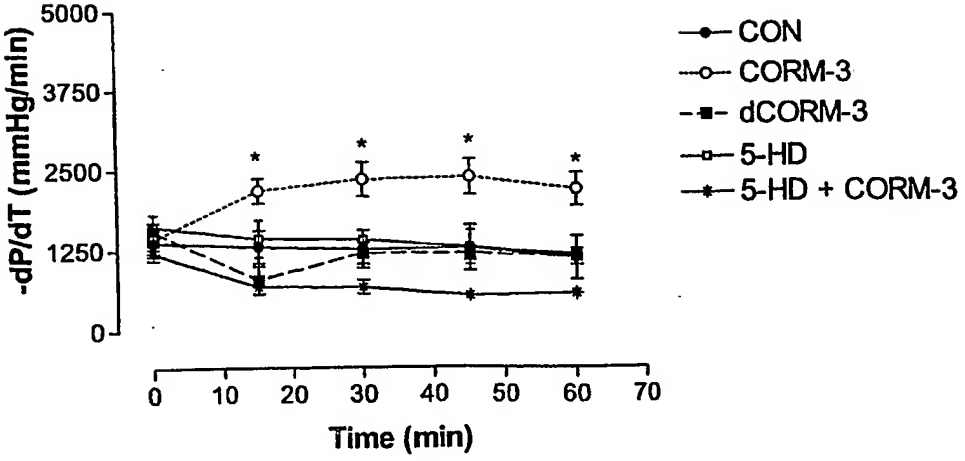
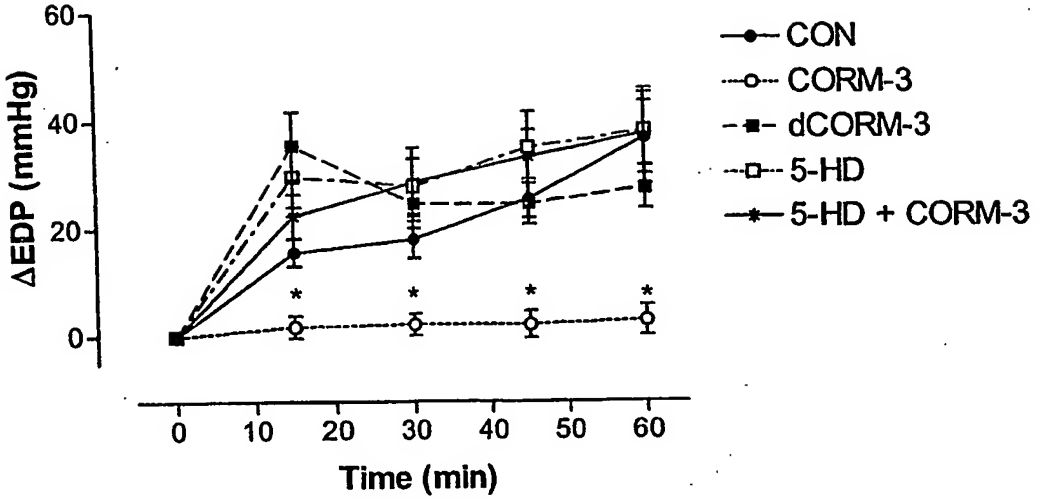

-dP/dT (mmHg/min)
5000
3750
2500
1250
0
0 10 20 30 40 50 60 70
Time (min)
CON
CORM-3
dCORM-3
5-HD
5-HD + CORM-3

FIGURE 2

A


60
40
20
0
ΔEDP (mmHg)
0
10
20
30
40
50
60
Time (min)
CON
CORM-3
dCORM-3
5-HD
5-HD + CORM-3
*
*
*
*

B

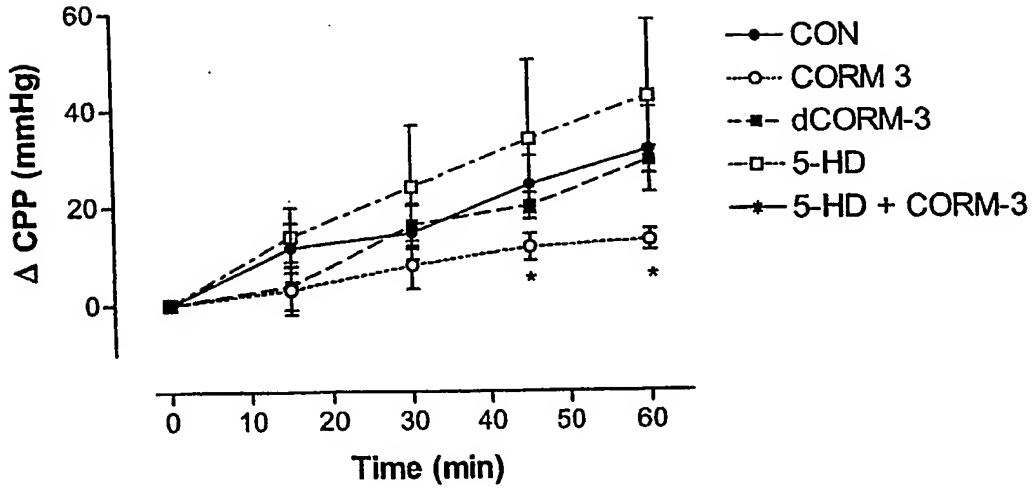
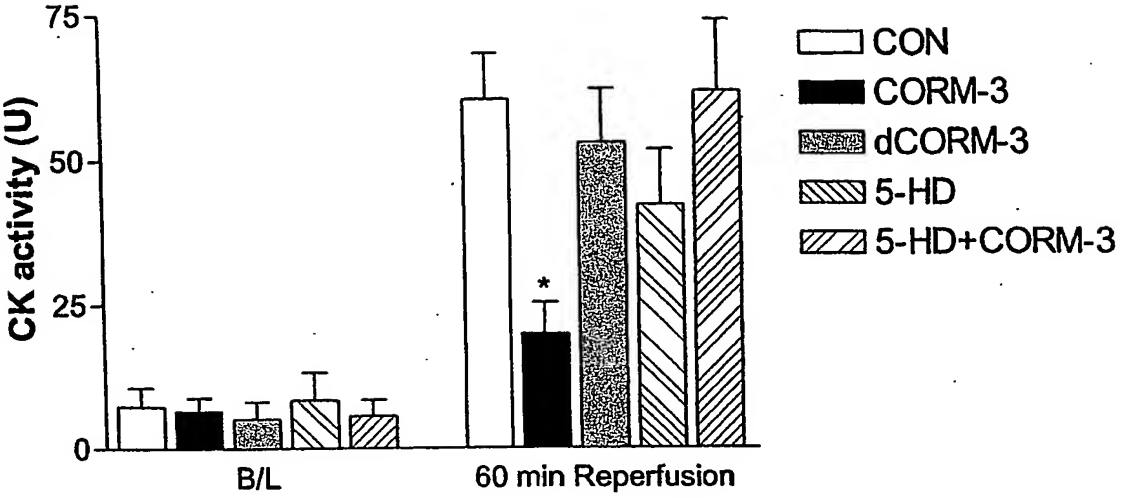
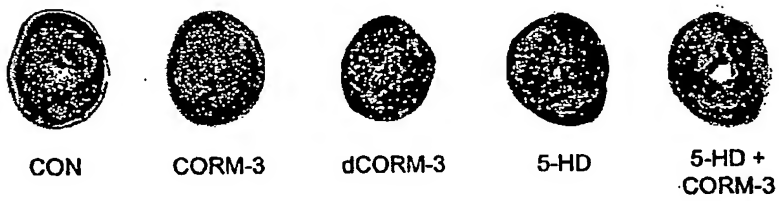

60
40
20
0
Δ CPP (mmHg)
0
10
20
30
40
50
60
Time (min)
CON
CORM 3
dCORM-3
5-HD
5-HD + CORM-3
*
*

FIGURE 3

A


75
50
25
0
CK activity (U)
*
B/L
60 min Reperfusion
CON
CORM-3
dCORM-3
5-HD
5-HD+CORM-3

B


CON
CORM-3
dCORM-3
5-HD
5-HD +
CORM-3

C

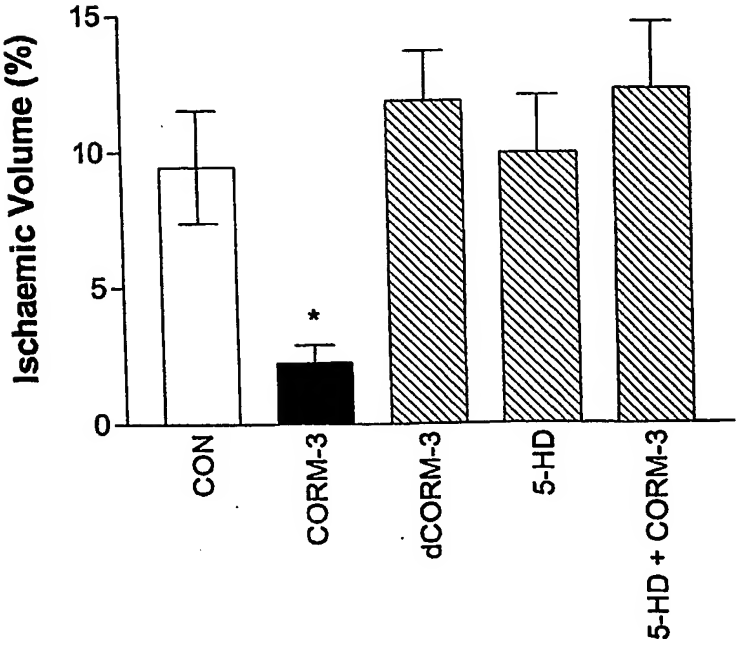

15
10
5
0
Ischaemic Volume (%)
*
CON
CORM-3
dCORM-3
5-HD
5-HD + CORM-3

FIGURE 4

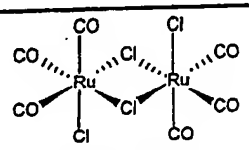
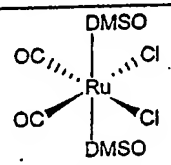
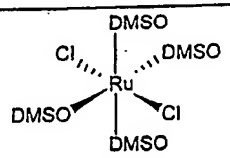
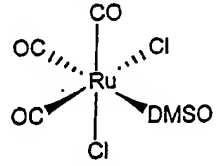
Compound	Structure	MW	CO Release (20 µmoles)				CO Release (40 µmoles)				NOTES
			0	10	20	30	0	10	20	30	
CO-RM-1		512	12.0 ±3.0	16.3 ±4.0	18.1 ±4.3	18.5 ±4.8	28.5 ±0.4	32.0 ±0.2	34.5 ±0.5	35.6 ±0.4	Soluble in DMSO
CO-RM-1a		384	7.2 ±0.6	8.6 ±0.3	8.0 ±0.4	7.5 ±0.4	16.9 ±0.6	18.4 ±0.3	17.3 ±0.3	16.7 ±0.2	Soluble in DMSO
Negative control		484	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	Soluble in H_2O
CO-RM-1b		334	6.4 ±1.2	7.3 ±0.6	8.2 ±0.1	8.7 ±0.3	11.7 ±0.8	13.7 ±0.9	14.0 ±1.1	14.4 ±0.6	Soluble in DMSO
CO-RM-10		(228)	2.6 ±0.6	9.8 ±0.3	12.7 ±0.1	13.8 ±0.9	8.6 ±0.7	21.0 ±1.1	24.4 ±1.0	26.3 ±1.2	Soluble in DMSO

FIGURE 5A

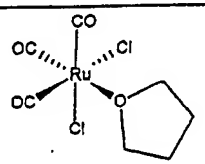
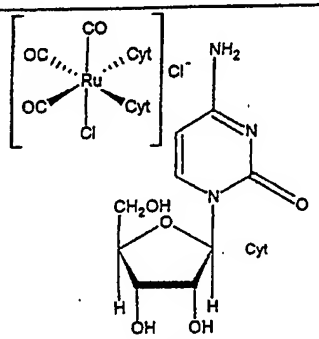
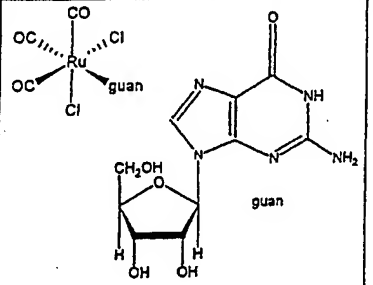
CO-RM-11 Ligand: THF		328	5.6 ±0.6	5.9 ±0.6	6.2 ±1.1	6.2 ±1.2	10.9 ±0.2	12.3 ±0.4	13.3 ±0.4	13.7 ±0.2	Soluble in DMSO
CO-RM-16 Ligand: Cytidine		742	N.D.	1.4 ±0.4	2.1 ±0.1	2.8 ±0.4	0.8 ±0.4	5.5 ±0.4	8.4 ±0.8	9.8 ±0.9	Soluble in H_2O
CO-RM-17 Ligand: Guanosine		539	5.9 ±0.1	8.2 ±0.4	8.5 ±0.3	8.6 ±0.4	11.5 ±0.4	15.0 ±0.4	15.6 ±0.4	16.2 ±0.3	Soluble in H_2O

FIGURE 5B

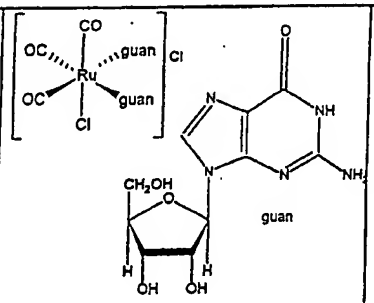
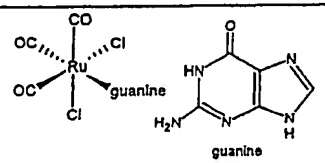
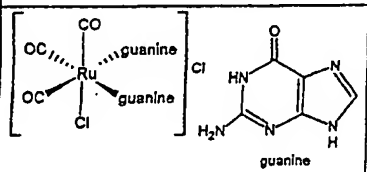
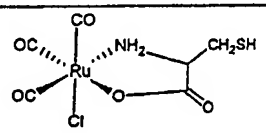
CO-RM-18 Ligand: Guanosine		822	10.1 ±0.9	14.3 ±0.4	14.1 ±0.5	13.5 ±0.4	25.4 ±1.0	29.5 ±1.5	29.5 ±1.4	28.7 ±1.3	Soluble in H_2O
CO-RM-22 Ligand: Guanine		407	0.1 ±0.1	0.8 ±0.3	1.0 ±0.3	2.3 ±0.1	0.7 ±0.1	1.9 ±0.1	2.3 ±0.1	2.4 ±0.1	Soluble in H_2O **PPT**
CO-RM-23 Ligand: Guanine		558	1.2 ±0.1	1.3 ±0.2	1.3 ±0.1	1.0 ±0.2	2.7 ±0.3	2.7 ±0.3	2.7 ±0.4	2.3 ±0.2	Soluble in H_2O **PPT**
CO-RM-26 Ligand: Cysteine		340.5	0.6 ±0.1	1.9 ±0.1	2.3 ±0.2	2.4 ±0.2	1.9 ±0.2	3.7 ±0.1	5.1 ±0.1	5.2 ±0.1	Soluble in H_2O

FIGURE 5C

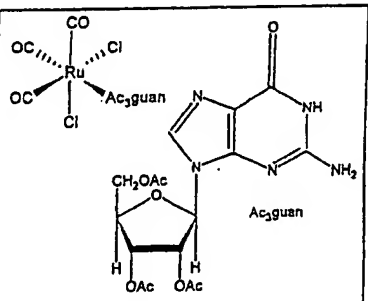
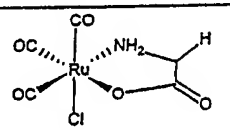
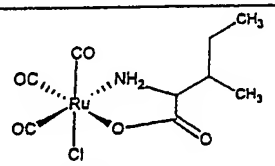
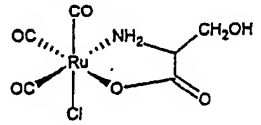
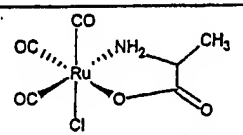
CO-RM-29 Ligand: Triacetyle-guanosine		665	1.4 ±0.7	4.5 ±0.1	5.0 ±0.1	3.2 ±0.1	8.3 ±0.6	11.7 ±0.3	12.4 ±0.1	10.6 ±0.4	Soluble in H_2O
CO-RM-3 Ligand: Glycine		294.5	14.2 ±0.6	17.8 ±0.7	14.3 ±0.7	12.9 ±0.7	25.2 ±1.5	24.4 ±1.0	23.8 ±0.6	23.2 ±0.3	Soluble in H_2O
CO-RM-38 Ligand: Isoleucine		350.5	3.2 ±0.2	4.4 ±0.1	4.0 ±0.2	3.0 ±1.7	7.6 ±1.3	8.3 ±1.2	7.5 ±1.1	7.3 ±1.1	Soluble in H_2O
CO-RM-39 Ligand: Serine		324.5	11.0 ±.03	12.8 ±.09	11.4 ±1.1	10.8 ±.07	24.2 ±1.5	24.6 ±1.4	22.0 ±1.0	21.9 ±1.2	Soluble in H_2O
CO-RM-40 Ligand: Alanine		308.5	9.1 ±1.1	11.9 ±0.4	11.1 ±.03	11.0 ±0.2	20.2 ±.06	21.3 ±.09	19.9 ±.09	19.6 ±.09	Soluble in H_2O

FIGURE 5D

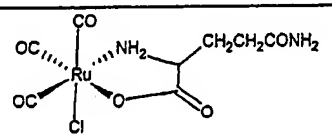
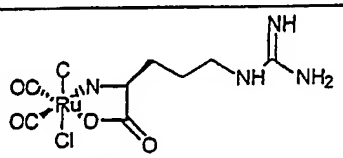
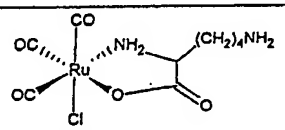
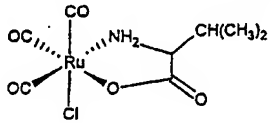
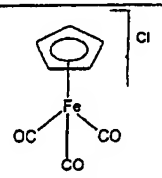
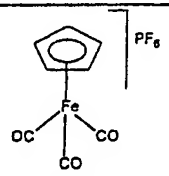
CO-RM-42 Ligand: Glutamine		365.5	8.9 ±0.4	11.1 ±0.4	12.1 ±1.4	10.1 ±0.3	21.4 ±2.1	21.8 ±2.2	20.6 ±2.0	20.0 ±1.8	Soluble in H_2O
CO-RM-43 Ligand: Arginine		393.5	9.4 ±1.4	11.9 ±0.5	12.3 ±0.7	11.0 ±0.3	18.3 ±.03	20.0 ±0.6	19.0 ±1.2	17.8 ±1.3	Soluble in H_2O
CO-RM-46 Ligand: Lysine		365.5	6.0 ±0.4	7.5 ±0.8	7.2 ±1.2	6.4 ±0.8	12.6 ±0.9	13.4 ±1.2	13.2 ±1.1	11.9 ±1.0	Soluble in H_2O
CO-RM-67 Ligand: L-valine		336.5	11.1 ±2.9	18.2 ±1.7	17.6 ±1.6	17.0 ±1.6	29.3 ±1.5	34.6 ±2.2	33.7 ±2.2	32.8 ±2.2	Soluble in H_2O
CO-RM-70		240	0.5 ±0.2	0.9 ±0.1	2.2 ±0.2	2.7 ±0.3	0.9 ±0.1	2.0 ±0.2	4.9 ±0.2	6.3 ±0.3	Soluble in DMSO **PPT**
CO-RM-71		350	1.5 ±0.2	2.3 ±0.3	3.1 ±0.4	3.7 ±0.4	3.4 ±0.1	5.4 ±0.3	6.9 ±0.3	7.6 ±0.4	Soluble in DMSO **PPT**

FIGURE 5E

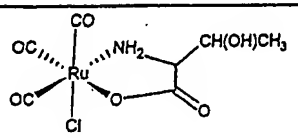
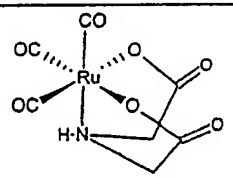
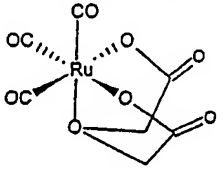
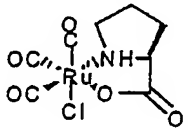
CO-RM-74 Ligand: L-Threonine		338.5	15.7 ±1.2	17.5 ±2.0	16.5 ±2.3	14.8 ±2.2	33.3 ±0.2	33.4 ±0.1	32.7 ±0.2	31.4 ±0.1	Soluble in H_2O
CO-RM-97		316	2.8 ± 0.6	7.0 ± 0.7	7.2 ± 0.9	6.6 ± 0.9	7.1 ± 0.5	14.3 ± 0.7	14.7 ± 0.8	13.6 ± 0.7	Soluble in H_2O
CO-RM-99		317	4.6 ± 0.6	8.1 ± 0.2	7.3 ± 0.3	5.5 ± 0.3	11.5 ±0.2	16.6 ± 0.2	16.0 ± 0.9	14.0 ± 0.2	Soluble in H_2O
CO-RM-H Ligand: L-proline		335	1.4 ± 0.3	4.7 ± 0.6	6.2 ± 0.8	6.3 ± 0.7	4.2 ±0.4	9.9 ± 0.2	12.5 ± 0.1	13.0 ± 0.1	Soluble in H_2O

FIGURE 5F